THE DEVELOPMENT OF LIPOSOME ENCAPSULATED CALCIUM PHOSPHATES FOR BONE REGENERATION

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

by

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2010

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CERTIFICATE OF AUTHORSHIP/ORIGINALITY

I, Kanthi Lewis, certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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Acknowledgements

First and foremost I would like to thank Dad. You always taught me to see through everything I commit to, no matter how tough it gets. Thanks for all the encouragement, love, support and belief you always had in me. Knowing how proud you have always been, even now that you are not here to remind me, always gives me strength to persevere.
Without you this would not have been possible, and this thesis is dedicated to your memory.

I would like to thank my wonderful supervisors Prof Besim Ben-Nissan, Dr Stella Valenzuela and Dr Louise Evans who have been incredibly supportive, and given me guidance these last 4 years. Always generous with your time and energy, I have learnt more than I ever thought possible, and I could not have asked for better supervisors.

Prof Racquel LeGeros, taking me on and mentoring me through my year at NYU, the experience was invaluable, and it has been an absolute privilege to learn from you.

I would also like to thank all the UTS staff particularly Mr Mark Berhkahn, Mr Ric Wurher, and Dr Ronald Shimmon, for help with the X-ray, Imaging, Mapping, Organic Synthesis and NMR, and Ms Linda Foley, Ms Era Koirala, all your help with ordering and generally make life as a PhD student much easier.

Dr Kristina Warton, Mr Nicholas Archer and Ms Stephanie Dowdell for all the assistance with the PCR, for always being there to help when I got to my wits end.

Dr Murray Killingsworth from Electron Microscopy Laboratory, South Western Area Pathology Service, for processing, sectioning and TEM imaging the liposomes.

Dr Dindo Mijares, I appreciate all your time and effort in the lab and helping me adjust.
Dr Yuexun Liu for your help with the PCR data analysis

Dr Catherine Kealley, you always listened and understood. Always a few steps ahead, you were a great source of advice, so often my shoulder to lean on, and a wonderful friend.
My fellow PhD students, Dr Julia Ting, and Dr Dakrong Pissuwan, who made the lab a much brighter place, and always willing to be a helping hand.

I would like to thank all my family and friends for all your love and support

ABSTRACT

Osteoporosis, a degenerative bone disorder, is one of the leading causes of morbidity in the elderly. Proper nutrition plays a role in the prevention and treatment of osteoporosis. Intake of calcium and Vitamin D are some of the most important nutritional factors, and supplementation remains the gold standard and first line of treatment for low bone mineral density and osteoporosis. Supplementation can prevent bone loss and reduce fracture risk.

This work set about to produce, characterise and encapsulate for direct delivery to the bone various micro and nano sized calcium based mineral compounds which may be beneficial to bone health, using the precipitation method and biomimetic processes.

Calcium phosphate mineral was produced and characterised, including hydroxyapatite (Hap), dicalcium phosphate dihydrate (DCPD), as well as multiphase and substituted calcium phosphates using biomimetic process. Standard simulated body Fluid (SBF) solution was modified, creating a high carbonate solution which better mimics the bone environment, and produces precipitates more similar to bone than traditional low carbonate SBF, as confirmed using Fourier Tansform Infrared Spectroscopy (FTIR) and X-Ray Diffraction (XRD).

The use of liposomes as a delivery vesicle for the calcium mineral was evaluated using FTIR, XRD, Electron Dispersive Spectroscopy, Mass Spectrometry Transmission and Scanning Electron Microscopy, and X-Ray Mapping. The calcium mineral from aqueous solutions and prepared HAp and DCPD was incorporated into the liposome.

Functional groups were synthesised based on a published structure used to target the bone marrow macrophage, and incorporation into liposomes was confirmed using Nuclear Magnetic Resonance Spectroscopy and FTIR. Preliminary cell culture studies showed no direct effect on osteoblast like Mg63 or Saos-2 cells or osteoclast resorption, measured by bone collagen release.

Macrophage response was explored using U937 cell line. Expression of TNF-α and IL-1, markers of inflammation, increased with liposome treatments compared to the negative control but decreased compared the positive control. The Mg63 cells given U937 supernatants showed liposomes increased OPG production, but this was regardless of mineralisation.

The calcium based mineral compounds were produced, characterised, successfully encapsulated using liposomes and functionalised to improve uptake at the bone site. This shows the potential to deliver calcium to the bone, however further work to inhibit inflammation and increase the calcium dose to elicit greater cell response is required before this approach can be developed as a treatment option.

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List of Abbreviations

Alkaline phosphatase (ALP)

Back scattered electron (BSE)

Bone mineral density (BMD)

Bovine serum albumin (BSA)

Calcium acetate ($Ca(Ac)_2$)

Colony stimulating factor-1 (CSF-1)

Dicalcium phosphate dihydrate (DCPD)

Environmnental Scanning Electron Microscope (ESEM)

Enzyme linked immunosorbent assay (ELISA)

Ethyleme diaminetetra acetic acid (EDTA)

Extracellular matrix (ECM)

Foetal Bovine Serum (FBS)

Fourier Transform Infrared Spectroscopy (FTIR)

Gaseous secondary electron (GSE)

Hormone replacement therapy (HRT)

Horseradish peroxidise (HRP).

Hydroxyapatite (Hap)

Inductively coupled plasma (ICP).

Institutional Animal Care and Use Committee (IUCAC)

Insulin like growth factor 1 (IGF-I)

Interferon-γ (IFN γ),

Interleukin (IL),

Latent TGF-β binding protein (LTBP)-1

Macrophage colony stimulating factor (MCSF)

Macrophage inflammatory protein-1 alpha (MIP-1a)

Mass spectrometry (MS)

Monocyte chemoattractant protein-1 (MCP-1)

Monoclonal antibodies (Mabs)

Propidium Iodide (PI)

Raloxifene (RAL)

Receptor Activator for Nuclear Factor κ B Ligand (RANK-L)

Repetitions per minute (RPM)

New York University (NYU)

Nuclear factor-jB ligand /RANK ligand (RANKL)

Nuclear magnetic Resonance Spectroscopy (NMR).

Osteoprotegerin (OPG)

Ovariectomized (OVX)

Parathyroid hormone (PTH),

Phosphate Buffered Saline (PBS)

Phosphatidycholine (PC)

Polymerase Chain Reaction (PCR)

p-nitrophenol (pNp)

p-nitrophenol phosphate (p-Npp)

Reactive oxygen species (ROS)

Ribonucleic Acid (RNA)

Scanning electron microscope (SEM)

Simulated body Fluid (SBF)

Small integrin-binding ligand N-linked glycoprotein (SIBLING) family

Tartrate-resistant acid phosphatase type 5b (TRAcP-5b)

Tetramethylbenzidine (TMB)

T helper 1 (T_H1)

Transmission electron microscopy (TEM).

Transforming growth factor (TGF)-β

Tumour-necrosis factor (TNF)

United States Department of Agriculture (USDA).

University of Technology, Sydney (UTS)

Width of Field (WOF)

X-ray Diffraction (XRD)

X-Ray Mapping (XRM)

7-dehydrocholesterol (7- DHC)

1,25-dihydroxyvitaminD_3 (1,25$(OH)_2D_3$).

1,2 distearoyl-sn-glycero-3-phospho-ethanolamine-N-[monomethoxy poly(ethylene glycol) 5000 (PEG-DSPE)

1,5 Dipalmmitoyl-L-glutamate-N-succinic acid (LGSA)

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Equation 2.1 Conversion of DCPD to HAp

$CaHPO_4 + Ca_4(PO_4)_2O$ Æ $Ca_5(PO_4)_3OH$

Equation 3.1 Bicarbonate buffering system

$CO_2(g) + H_2O \Leftrightarrow CO_2\ (aq) + H_2O$ (1) dissolved CO_2

$CO_2(aq) + H_2O \Leftrightarrow H_2CO_3$ (2) carbonic acid

$H_2CO_3 \Leftrightarrow H^+ + HCO_3^-$ (3) bicarbonate pKa_1 =6.35 at 25°C

$HCO_3^- \Leftrightarrow H^+ + CO_3^{2-}$ (4) carbonate pKa_2=10.33at 25°C

Equation 3.2 Frequency of molecular Vibrations

$$\upsilon = \frac{1}{2\pi}\sqrt{\frac{k(m_1 + m_2)}{m_1 m_2}}$$

Equation 3.3 Relationship between wavelength, incident radiation and diffraction

$n\lambda = 2d\sin\theta$

Equation 4.1 Calculation of Area of PEG dispersion

$APEG = \pi R_F^2$

Equation 4.2 Definition of Florey radius

$RF = N^{3/5}a$

Equation 4.3 Percentage of lipid surface covered with PEG

$R = A_{PEG}$ x M / A_{lipid}

Equation 5.1 Calculation of ALP activity

$$IU = \mu mol/(L \cdot \min)$$

$$= \frac{(ODsample_t - ODsample_0) \cdot 1000 \cdot \mathrm{Re}actionVol}{t \cdot \epsilon \cdot l \cdot samplevol}$$

$$= \frac{(ODsample_t - ODsample_0) \cdot \mathrm{Re}actionVol}{(ODcalibrator - ODH_2O) \cdot SampleVol \cdot t}$$

Equation 7.1. Change between gene of interest and housekeeping gene (HKG)

Gene 1 Ct – HKG Ct = ΔCt

Equation 7.2. Sample change compared to control sample

Sample ΔCt – Control ΔCt = X

Equation 7.3. Find fold increase, as each Cycle represents and exponential increase

Fold increase (or decrease) = Power (X,2)

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